Cellular and molecular mechanisms of hepatic fibrogenesis

Fabio Marra


INTRODUCTION

Progressive accumulation of fibrillar extracellular matrix (ECM) in the liver is the consequence of reiterated liver tissue damage due to infective (mostly hepatitis B and C viruses), toxic/drug-induced, metabolic and autoimmune causes and the relative chronic activation of the wound healing reaction. The process may result in clinically evident liver cirrhosis and hepatic failure. Cirrhosis is defined as an advanced stage of fibrosis, characterized by the formation of regenerative nodules of liver parenchyma that are separated by and encapsulated in fibrotic septa and associated with major angio-architectural changes. While millions of patients worldwide are affected by chronic liver diseases potentially leading to cirrhosis, only a minority (~25-30%) are likely to develop significant fibrosis and cirrhosis. This is particularly true for chronic hepatitis due to HCV infection, whose prevalence is predicted to peak between the years 2010 to 2015. In recent years, attention has been focused on «non-alcoholic fatty liver disease» (NAFLD) and in particular on «non-alcoholic steatohepatitis» (NASH). The occurrence of NASH is associated with progressive fibrosis and cirrhosis in a high percentage of patients (up to 50%). Considering that, in industrialized countries, NASH affects 3% of the general population and 20-45% of obese patients, it becomes clear that this clinical entity represents a major health problem. Independently of the etiology, liver cirrhosis is the most common non-neoplastic cause of death among hepatobiliary and digestive diseases, both in the United States of America and Europe. In addition, this condition is largely associated with primary liver cancer, with a further increment in the relative mortality rate.

In general terms, there are distinct patterns of fibrotic development, related to the underlying disorders causing the fibrosis. Biliary fibrosis, due to the co-proliferation of reactive bile ductules and periductular (myo)fibroblast-like cells at the portal-parenchymal interface, tends to follow a portal to portal direction. This leads to the formation of portal-portal septa surrounding liver nodules, where the central vein and its connections with the portal tract are preserved until late stages. In contrast, the chronic viral hepatitis pattern of fibrosis is considered the results of portal-central (vein) bridging necrosis, thus originating portal-central septa. In addition, this form of fibrogenic evolution is characterized by the presence of interface hepatitis and development of portal to portal septa and septa ending blind in the parenchyma, by rapid derangement of the vascular connections with the portal system (early portal hypertension). The so-called central to central (vein) form of fibrogenic evolution is in general secondary to venous outflow problems (e.g.}

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chronic heart failure) and is characterized by the development of central to central septa and reversed lobulations. Finally, a peculiar type of fibrosis development (pericellular/sinusoidal) is observed in alcoholic and metabolic liver diseases (e.g. NASH), in which the deposition of fibrillar matrix is concentrated around the sinusoids (capillarization) and around groups of hepatocytes (chickenwire pattern). These different patterns of fibrogenic evolution are related to different factors and particularly: a) the topographic localization of tissue damage, and b) the relative concentration of pro-fibrogenic factors, and 3. the prevalent pro-fibrogenic mechanism(s). In addition, these different patterns imply the participation of different cellular effectors of the fibrogenic process.

CELLULAR EFFECTORS OF HEPATIC FIBROGENESIS

As the liver becomes fibrotic, there are both quantitative and qualitative changes in the composition of the hepatic ECM. The total content of collagenous and noncollagenous components increases 3-5-fold, accompanied by the shift in the type of ECM in the subendothelial space from the normal low-density basement membrane-like matrix to interstitial type matrix containing fibel-forming collagens. These quantitative and qualitative changes in the composition of ECM, in addition to their mechanical and physical implications, contribute to the formation of a new biochemical environment. Indeed, each ECM component has the ability to modulate cell growth, migration, gene expression, and other important cellular functions directly by interaction with cell adhesion molecules and, indirectly, by functioning as a biologic reservoir for pro-inflammatory and fibrogenic mediators in their active or inactive forms. The cellular source of connective tissue components in fibrotic liver has been controversial for many years, with some elements of controversy still remaining. Among other cell types potentially involved in the abnormal progressive deposition of fibrillar ECM, HSC have received much attention, perhaps due to the possibility to isolate these cells from liver tissues with a relatively high purity. Consequently, most of the present knowledge on the cell and molecular biology of hepatic fibrosis derives from in vitro studies employing culture activated HSC isolated from rat, mouse or human liver. Regardless, it is now evident that distinct ECM-producing cells, each with a distinct localization and a characteristic immunohistochemical and/or electron microscopic phenotype, are likely to contribute to liver fibrosis. These include: fibroblasts and myofibroblasts of the portal tract, smooth muscle cells localized in vessel walls, and myofibroblasts localized around the centrallobular vein. It is now evident that the relative participation of these different cell types is dependent on the development of distinct patterns of fibrosis. Major efforts are currently made in order to identify and characterize the origin of the different cell types responsible of the fibrogenic process. By employing cell identification markers, three populations of ECM producing cells can be identified: a) septal myofibroblasts, present in the inner part of fibrous septa, expressing a panel of cell markers identical to portal myofibroblasts; b) activated HSC, located in capillarized sinusoids adjacent to expanded portal tracts, and c) interface myofibroblasts, located at the edge of fibrous septa. This last population presents with an expression profile intermediate between portal myofibroblasts and activated HSC. It is conceivable that interface myofibroblasts derive from activated HSC recruited at the site of active fibrogenesis, where there is high level of cell damage, extracellular matrix degradation by gelatinases and inflammatory infiltration. In addition, preliminary reports suggest that bone marrow-derived stromal cells recruited at the site of liver injury could contribute to this population of fibrogenic cells.

PRO-FIBROGENIC MECHANISMS

Some of the emerging profibrogenic mechanisms for the fibrogenic evolution of CLDs will be summarized below. In general, fibrosis should be regarded as an alteration of the process of chronic wound healing. This process, which is highly efficient in the presence of a single acute tissue insult, leads to progressive scarring when tissue damage is chronic. In other terms, deposition of fibrillar matrix rather then organized tissue regeneration becomes the best option in order to maintain tissue continuity. Hepatic fibrogenesis is characterized by the following key features: a. the persistence of hepatocellular/choanal/endothelial damage with variable degree of necrosis and apoptosis; b. a complex inflammatory infiltrate including mononuclear cells and immunocompetent cells; c. the activation of different types of ECM-producing cells (HSC, portal myofibroblasts, etc.) with marked proliferative, synthetic and contractile features; d. marked changes in the quality and quantity of the hepatic ECM associated with very limited or absent possibilities of remodeling and regeneration. Inflammation

One of the first steps in tissue repair is the recruitment of inflammatory cells in order to neutralize possible infectious agents and to remove the necrotic tissue. In this phase of the process, HSC are recruited at the site of injury in order to synthesize and secrete ECM components. Recruitment and proliferation of HSC is under the control of soluble factors secreted by the cells of the inflammatory infiltrate. However, in the presence of reiterated tissue damage, HSC secrete several chemokines thus becoming a site of amplification and chronic organization of the inflammatory infiltrate. Monocyte chemotactic protein-1 (MCP-1) is the most prominent chemotactic factor secreted by chronically activated HSC and it Proinflammatory cytokines such as interleukin-1, tumor necrosis factor-
alpha and interferon-γ have been shown to be strong stimulators of gene and protein expression of MCP-1 in HSC. The exposure of HSC to soluble mediators that may potentially affect their pro-fibrogenic role has represented a key area of investigation in the last decade. It is important to stress that exposure to these mediators, genetically defined as "inflammatory", may be time-limited or chronically present according to the nature, extent and reiterative of parenchymal damage. A pivotal profibrogenic mechanism operated by infiltrating cells, such as inflammatory cells, is the synthesis and release of soluble factors playing a biological role on HSC. Consolidated experimental evidence suggests that two polypeptide growth factors, namely platelet-derived growth factor (PDGF) and transforming growth factor-β1 (TGFβ1), greatly contribute to the profibrogenic role of HSC. TGFβ1 binds to the superfamily of receptors comprising a large number of structurally related polypeptide growth factors, each capable of regulating different arrays of cellular processes including cell proliferation, differentiation, motility, adhesion and death, thereby playing a prominent role in the development, homeostasis and repair of all tissues in organisms. Members of the TGFβ cytokine family initiate signalling through their interaction with heteromeric type II TGFβ receptors, which propagate signals downstream through phosphorylation of cytoplasmic mediators of the receptor-regulated Smad family. Upon activation, a Smad2/3-Smad4 complex will translocate to the nucleus where they are involved in the regulation of transcription factors of TGFβ, target genes such as collagen type I. In many fibrogenic diseases abnormal accumulation of ECM proteins is associated with increased expression of the TGFβ family receptors. Established evidence indicates that this growth factor plays a multiple role in hepatic fibrogenesis. In activated HSC, TGFβ1 induces a strong and consistent up-regulation of the genes encoding for fibrillar collagens (particularly collagen type I and collagen type III) and other ECM components. In addition, TGFβ1 induces a down-regulation of the gene encoding for fibronectin (a collagen type I and collagen type III) and other ECM components. Inactivation of the TGFβ1 receptor Alk5 or the expression of dominant-negative TGFβRII in mouse liver results in reduced ECM accumulation and decreased fibrogenesis. The ability of TGFβ1 to activate the p38 MAPK pathway has been implicated in the regulation of ECM production and fibrosis. TGFβ1 signal transduction is initiated by binding to the receptor, which induces autophosphorylation and the activation of downstream signalling molecules such as Grb2 which recruits mSos, followed by Ras activation and Erk translocation to the nucleus where it will increase the expression of c-fos, a transcription factor, necessary for PDGF-induced mitogenesis, chemotaxis and cell survival.

Oxidative stress

When chronic liver injury is not clearly associated with an abundant inflammatory infiltrate, other soluble agents may sustain the activation of HSC through pathways that are specific for a particular type of damage. Evidence for oxidative stress has been detected in almost all the clinical and experimental conditions of CLDs with different etiology and fibrosis progression rate. Evidence in association with decreased antioxidant defenses. As already proposed for atherosclerosis and chronic degenerative diseases of CNS, oxidative stress-related molecules such as reactive oxygen intermediates (ROI) and reactive aldehydes, may act as mediators able to modulate tissue and cellular events responsible for the progression of liver fibrosis. In alcoholic liver injury, for example, acetaldelyde, the main metabolite of ethanol, is able to increase gene transcription and synthesis of different ECM components in activated HSC. In addition to acetaldelyde, products of lipid peroxidation generated by exposure to ethanol or the production of iron overload may also perpetuate HSC activation. Along these lines, stimulation of lipid peroxidation or exposure to 4-hydroxynonenal (4-HNE), a highly reactive aldehydic end-product of lipid peroxidation, increases procollagen I gene expression in activated human HSC. Inflammation and oxidative stress are tightly linked together. In a recent study, we demonstrated that interfering with the mechanisms of inflammatory cell recruitment as observed in MCP-1 knock-out mice, limits the generation of intrahepatic oxidative stress. On the other hand, some types of leukocytes have been shown to counter-regulate the development of fibrosis. Depletion of NK cells worsens matrix accumulation, due to the fact that this cell type induces programmed cell death of activated HSC.

Derangement of the epithelial-mesenchymal interaction

Cholangiopathies are progressive liver disorders representing a major cause of chronic cholestasis both in adults and children. Both bile duct proliferation and ductular metaplasia are associated with profound changes in the surrounding mesenchymal cells and extracellular matrix. It is likely that at least in the early phases, ECM-producing cells other than HSC are primarily involved, whereas HSC become subsequently involved when proliferating bile ducts tend to invade lobular areas. It is still unclear whether the changing epithelial phenotype directly in-
duces an alteration in portal mesenchymal cells and ECM or whether the epithelial cell changes are induced by modifications in ECM. Cytokines and proinflammatory mediators, released in the portal spaces, likely contribute to these processes by activating fibrogenesis, stimulating apoptotic and proliferative responses, damaging the peribiliary circulation, increasing the expression of histocompatibility antigens in cholangiocytes and by altering the transport functions of the epithelium. An emerging concept is that bile duct epithelial cells are active participants in inflammatory diseases and, in pathologic conditions, secrete proinflammatory and chemotactic cytokines, such as IL-6, TNFα, IL-8, and MCP-1, together with growth factors able to activate mesenchymal cells and matrix production (ET-1, PDGF-BB, TGFβ1, CTGF)49-51. These mediators, either released in the portal spaces by immune cells, macrophages and mesenchymal cells or produced by the epithelium itself, may have profound effects on epithelial cell function. Accordingly, several lines of evidence suggest that «activated» cholangiocytes play an active role in stimulating the fibrogenic response, through an extensive cross-talk with portal fibroblasts/myofibroblasts and HSC. It is very relevant that this close association between bile duct proliferation and mesenchymal activation is present also in cholangiocarcinomas, a group of neoplasms frequently characterized by a strong desmoplastic reaction.

**Adipokines**

This group of cytokines produced by adipose tissue is believed to play a role in nonalcoholic steatohepatitis (NASH), that develops in the presence of an excess of fat. Leptin is a hormone produced by adipocytes, that regulates food intake via actions on the hypothalamus. Different groups have provided compelling in vivo evidence for the pro-fibrogenic action of leptin in rodents42-44. Injection of leptin during acute and chronic intoxication results in a marked upregulation of the expression of type I procollagen and transforming growth factor-β1, a key pro-fibrogenic cytokine. This finding was followed by the demonstration that scarring is reduced in fa/fa rats or ob/ob mice chronically exposed to thioacetamide. These studies also confirmed that the expression of pivotal profibrogenic mediators is limited in the absence of leptin signaling. The pro-fibrogenic action of leptin depends, at least in part, on a direct effect on HSC, which express functional leptin receptors and are directly responsive to leptin. Incubation of HSC with recombinant leptin stimulates mRNA and protein expression of type I procollagen, potentiates the effects of TGFβ1, and up-regulates expression of the tissue inhibitor of metalloproteinase (TIMP)-1, thus blocking collagen degradation45-47. Moreover, leptin is a mitogen and a survival factor for activated HSC, and limits their apoptosis, resulting in an increase in the number of fibrogenic cells, and induces secretion of pro-inflammatory chemokines such as MCP-1, via activation of nuclear factor-κB. Finally, a recently-identified activity of leptin on fibrogenic cells is the induction of vascular endothelial growth factor, one of the most potent inducers of neovessel formation, via oxygen-independent activation of hypoxia-inducible factor 1α, a master switch of the angiogenic response48. These data suggest that leptin may be an effector of the increased fibrogenesis observed in obese patients.

Adiponectin is a recently identified protein that is predominantly, but not exclusively, by the adipose tissue. It circulates at high levels in the bloodstream, representing one of the main plasma proteins. Adiponectin is considered a major determinant of the sensitivity to insulin, acting at different sites of glucose metabolism, including liver, muscle, and fat itself. Experimental data also show that administration of recombinant adiponectin ameliorates metabolic derangements and liver damage in mouse models of alcoholic and nonalcoholic hepatitis51. Thus, adiponectin may block the development of fibrosis limiting hepatic damage. More recently, a direct antifibrogenic action of adiponectin has been demonstrated in animals undergoing toxic liver damage52, a condition independent of deranged metabolism. In addition, a balance between the biology of leptin and that of adiponectin seems to take place in stellate cells53. Adiponectin’s effects are mediated by two receptors, known as AdipoR1 and AdipoR253, and at least some of the metabolic effect of adiponectin are dependent on receptor-mediated activation of AMP-dependent protein kinase. However, the contribution of the different receptor isoforms and/or AMP-dependent kinase to the antifibrogenic effects of adiponectin has not yet been elucidated. The emerging biology of adiponectin make this molecule a very appealing target for future studies in NASH and other liver diseases.

Other adipokines are possibly implicated in the fibrogenic process. Resistin contributes to insulin resistance in rodents, but its metabolic effects in humans are still uncertain. Recent evidence obtained in our laboratory indicates that resistin modulates the biology of human HSC inducing a pro-inflammatory phenotype. In addition, like reported for other adipokines, the expression of resistin is detectable in liver tissue, especially in conditions of fibrosis54.

**Renin-angiotensin-aldosterone system**

The renin-angiotensin system is another pivotal player in the pathogenesis of liver fibrosis. Exposure of fibrogenic cells to angiotensin II mediates key biological actions involved in hepatic tissue repair, including proliferation, infiltration of inflammatory cells, and collagen synthesis55. Activated HSC express all components of the renin-angiotensin system, and the autocrine effects of Ang II are mediated by activation of NADPH oxidase55,56. Blockade of the renin-angiotensin system attenuates fibrosis development in different experimental models of chronic liver injury56. In addition, aldosterone antagonists also have a direct antifibrogenic action57. Interfering with the renal-
angiotensin system is therefore a very promising strategy to prevent fibrosis progression in chronic liver diseases, and controlled clinical trials are under way.

**Nuclear hormone receptors**

A number of studies has recently suggested that antidiabetic thiazolidinediones (TZD), or «glitazones» may represent a possible novel pharmacological treatment for liver fibrosis. TZDs are employed for the treatment of insulin resistance in patients with type 2 diabetes and are selective ligands for the nuclear transcription factor peroxisome proliferator-activated receptor (PPAR)γ. PPARγ is expressed in quiescent HSC and its abundance and/or transcriptional activity decreases along the activation process that accompanies the acquisition of fibrogenic properties. More important, exposure of HSC to PPARγ ligands, including different glitazones, reverts most features of the activated phenotype of HSC. In these cells, PPARγ activation reduces the expression of interstitial collagens and other matrix proteins, downregulates the ability to proliferate and migrate in response to PDGF, blocks the secretion of pro-inflammatory chemokines such as monocyte chemotactant protein-1, and induces apoptosis. In addition, daily intragastric administration of rosiglitazone or pioglitazone, started at the same time as injury, leads to a marked reduction of fibrotic tissue accumulation and fibrogenic cell proliferation. These data provided compelling in vivo evidence supporting an antifibrogenic role of thiazolidinediones, although recent data suggest that administration of these drugs may be less effective if started after the onset of injury.

**Other mechanisms**

Recently, different groups have reported the biological effects of proteins of the hepatitis C virus (HCV) on cultured HSC. A number of different approaches have been used, including incubation with recombinant proteins of the envelope, adenoviral-mediated overexpression of HCV proteins or exposure to conditioned media of hepatocyte-like cells expressing the HCV replicon. Exposure to HCV proteins induces increased expression of extracellular matrix and ECM-regulating cytokines (collagens, TGF, CTGF) and up-regulation of MMP-2, an index of HSC activation. In addition, down-regulation of fibrolytic matrix-metalloproteinases (e.g. MMP-1), induction of ROS generation, and up-regulation of pro-inflammatory cytokines have been described. This represents a novel area of investigation linking fibrogenesis and viral hepatitis. The cannabinoid system comprises an additional group of mediators that have been recently implicated in the regulation of fibrogenesis. Anandamide, a lipid mediator, efficiently induces necrosis in activated HSCs, but not hepatocytes. In addition, interference with cannabinoid receptors leads to differential results. Knock-out of CB2 is associated with increased fibrosis, demonstrating that CB2 activation triggers antifibrotic pathways. In contrast, animals deficient for CB1 have lower accumulation of scar tissue, indicating a pro-fibrogenic action for this receptor. These results may have a relevance ion consideration of the availability of rimonabant, a selective CB1 blocker, that has been proposed as a therapy for the metabolic syndrome.

**Future directions**

The increasing knowledge on the pathogenesis of hepatic fibrosis has led to important changes in the clinical interpretation of this phenomenon. Firstly, the need of an accurate and effective monitoring of the fibrotic progression of chronic liver diseases and of the effectiveness of the currently proposed treatments has become an impellent need. Moreover, the identification of the genes involved in the progression of liver fibrosis would hopefully lead to the establishment of prognostic markers indicating a faster progression of fibrogenic chronic liver diseases. Along these lines, several ongoing studies are addressing the relevance of gene expression and/or gene polymorphisms in defined subset of patients. Finally, novel profibrogenic pathways are being elucidated, leading to new approaches for antifibrotic treatments.

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